An evolving architecture: The past, present & future of indoor microbiology

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Introduction

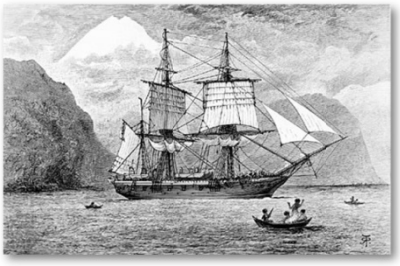
In this presentation I will discuss the microbiology of the built environment, its history, current state of knowledge, and its future, with specific reference to the limitations of sampling and analytical methods. In turn, I'll outline how these factors have influenced our knowledge of the microbiology of the built environment as a determinant of health.

Part I – Where we've been

I chose to begin this talk in the past. It might seem odd to begin a discussion essentially about the future of aerobiology and indoor microbiology with a discussion of work done well over a century ago.

However, my goal in briefly tracing aerobiology to its roots is to provide you with a clear sense of how and why our modern sampling tools have developed, and to demonstrate how today we really have crossed the precipice of a technological revolution in the science of biology that is changing fundamentally how we study life, and in the process, overturning many of our long-held ideas.

What's happening in biology now is not unlike the revolution Einstein brought to the deeply-entrenched Newtonian physics of the early 20th century through his transformative ideas on relativity. But I'm getting ahead of myself.



The modern science of aerobiology began in an unlikely place, on this ship, the HMS Beagle, a Cherokee-class 10-gun brig-sloop of the British Royal Navy that circumnavigated the southern hemisphere during a series of 3 exploratory voyages. The event I'm referring to happened during her second voyage, a 5-year survey chiefly of South America, that began in late 1831.

The young naturalist Charles Darwin had been taken aboard as the survey biologist. Darwin was a keen observer of life and all its patterns. These observations ultimately culminated in his pioneering evolutionary theory. But Darwin's great fame for authoring "On the Origin of Species" overshadowed his role as a key figure in the history of aerobiology, as I'll discuss.

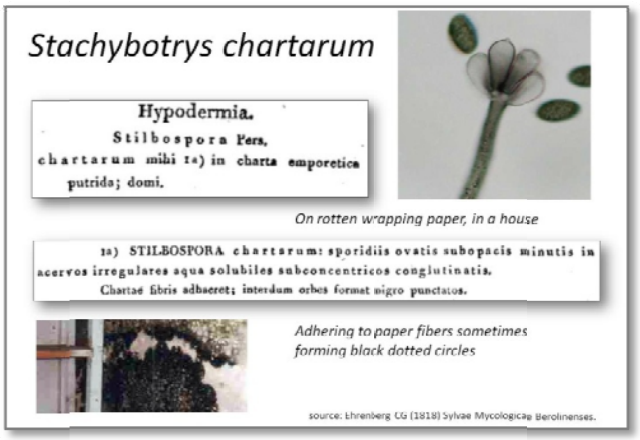
It was during Darwin's voyage on the Beagle that the ship twice passed through the high seas of the Cape Verde archipelago, some 500 miles off the African coast that Darwin noticed an accumulation of orange dust on the deck and riggings of the ship.

Darwin believed it to be volcanic ash or iron-rich dust carried aloft by the northeast trade winds from the Sahara desert thousands of miles to the east.

Darwin collected 5 samples of the dust and sent them to Christian Gottfried Ehrenberg, a professor at the University of Berlin. Ehrenberg was a fellow naturalist who, like Darwin, shared an expertise in tropical corals.

Also like Darwin, Ehrenberg had spent time at sea as a survey naturalist on expeditions led by the famous German explorer, Baron Alexander von Humboldt.

And although you may not realize it, you're likely already familiar with some of Ehrenberg's work.



Ehrenberg's chief expertise was in the study of microscopic life forms, and his doctoral thesis in mycology in 1818 dealt with the fungi of Berlin. In it, he first described from decaying wrapping paper the now-notorious microfungus, *Stachybotrys chartarum*.

Ehrenberg prepared microscopic mounts from Darwin's samples by combining equal portions of dust and glucose syrup. When Ehrenberg examined the preparations, what he found was remarkable and unprecedented. Many of the particles contained in the dust were (or had at one time been) living organisms. From his studies of these materials, Ehrenberg wrote the first treatise on microscopic biological particles in the atmosphere – evocatively entitled "Passatstaub und Blutregen" – "Trade Dusts and Blood Rain".

Passatstaub und Blutregen.

Ein grofses organisches unsichtbares Wirken und Leben in der Atmosphäre.

Von

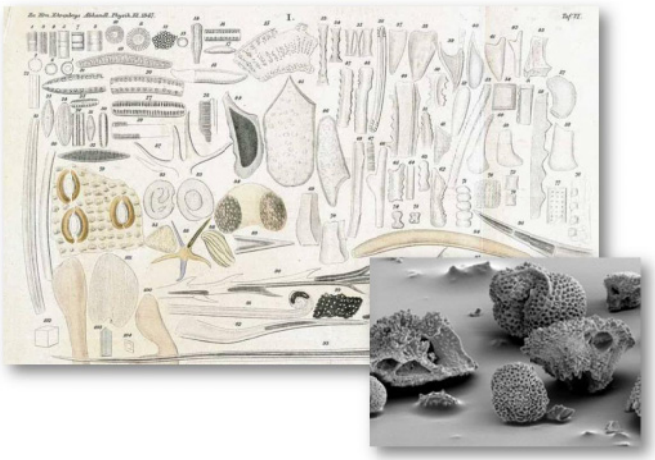
H^n EHRENBERG.

I.

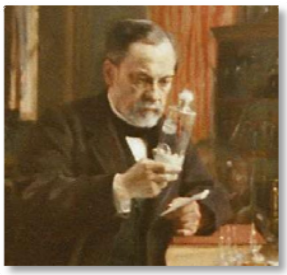
Ueber einen die ganze Luft längere Zeit trübenden Staubregen im hohen atlantischen Ocean in 17° 43' N. B. 26° W. L. und dessen Mischung aus zahlreichen Kieselthieren. (*)

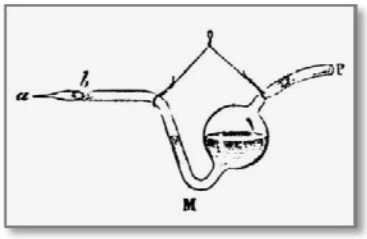
Herr Darwin, der bekannte verdienstvolle englische Reisende und Schriftsteller über die Corallenriffe, erzählt in seinem Reiseberichte, dafs auf den Capverdischen Inseln und auch im hohen Meere jener Gegend bei seiner Anwesenheit daselbst beständig ein feiner Staub aus der Luft gefallen sei und auch die Schiffe, welche 380 Seemeilen vom Lande entfernt waren, wurden, seinen brieflichen Mittheilungen zufolge, davon betroffen. Der Wind wehte damals von der afrikanischen Küste her. Von dem Staube aus der hohen See, welcher in so grofser Entfernung vom Lande auf das Schiff niederfiel, hat Herr Darwin eine Probe meiner mikroskopischen Prüfung übergeben. Es wurde bisher dieser dort häufige Staub, seiner gelbrothen Farbe halber, allgemein für eine vulkanische Asche gehalten. Die mikroskopische Analyse hat zur Klarheit ergeben, dafs ein namhafter Theil, vielleicht ⅟ der Masse, aus sehr verschiedenen kieselschaligen Polygastricis und kieselerdigen bekannten terrestrischen Pflanzentheilen besteht, wie folgt:

The organisms Ehrenberg found were dominated by the calcified shells of coccoliths and foraminifera, planktonic plants and animals whose populations we now know surge in response to the iron-rich desert dust that is periodically dumped into the ocean by the trade winds. The organisms in his illustrations remain recognizable today, and they include many pollen grains and fungal spores.



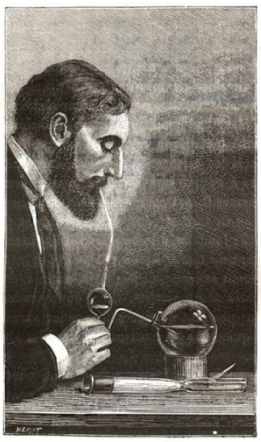
It took nearly a decade after the publication of Ehrenberg's dust studies for the French chemist Louis Pasteur to realize the full implication of





microscopic life in the air. Unlike Ehrenberg, Pasteur cultured airborne microbial cells directly in swan-necked flasks containing boiled broth. Although Pasteur's chief interest was in understanding the processes of fermentation key to the manufacture of beer and wine, he also sampled air from a range of building types and outdoor environments. Through these experiments, Pasteur demonstrated unambiguous proof of the germ theory of disease, that life is responsible for creating life, and forever overturned the idea that decay and disease arose spontaneously.

The revolutionary idea that airborne microscopic life forms or "germs" could cause disease led many scientists to begin sampling the air in search of the agents of the prevailing diseases of the day. And one of the most important then, was the diarrheal disease, cholera, which had devastated many European cities in waves over the 18th and 19th centuries.




LES

ORGANISMES VIVANTS

DE L'ATMOSPHÈRE,

PAR M. P. MIQUEL,

PARIS,

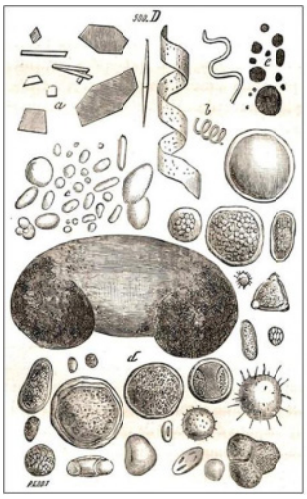
GAUTHIER-VILLARS, IMPRIMEUR-LIBRAIRE

1883

In Paris, the search for an airborne pathway of cholera transmission was led by Pierre Miquel, a pharmacist by training who later studied medicine, culminating in a thesis on living organisms of the atmosphere. Miquel was a contemporary of Pasteur, and worked at the Montsouris meteorological observatory.

Miquel developed what is recognized to be the first known volume-based microbial air sampler. Miquel's apparatus operated at a flow rate of 330 mL per minute, and ran for 2 days in order to collect the sample. Miquel's sampling pump didn't use electricity: instead, this elegant device relied on the vacuum created by the descent of water through a p-trap. Amazingly, using only 4 litres of water, Miquel's pump was able to collect an entire cubic meter of air. Miquel then analysed his captured particles using microscopy in a manner that differs little from today's spore trap analysis.

Miquel conducted extensive sampling of the air throughput Paris, he sampled in hospitals and other buildings, and even in the sewer system. But the water-borne transmission pathway of cholera was unknown to Miquel; accordingly, the cholera vibrio remained elusive throughout his air sampling. Miquel did succeed in establishing an extensive catalogue of airborne life, some familiar, others unknown, and demonstrated interesting characteristics of their responses to seasonal change. With this work, he laid the foundation for modern aerobiology.



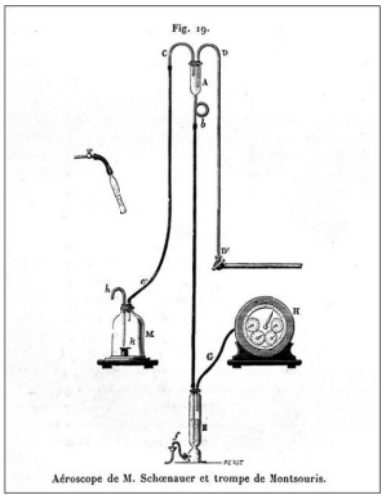


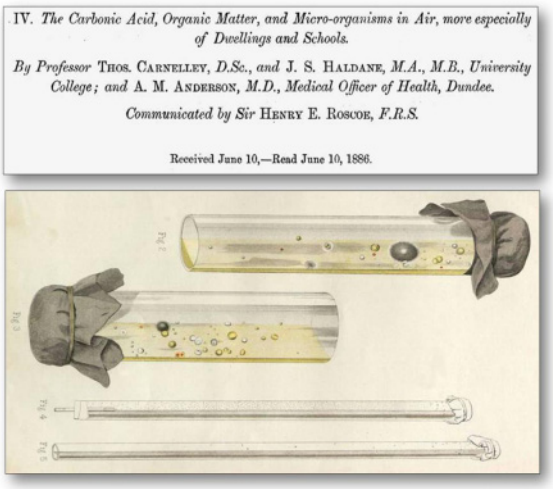
Fig. 19.

Aéroscope de M. Schœnauer et trompe de Montsouris.

Despite the failure of air sampling to clarify the epidemiology of cholera, others recognized its potential value to investigate associations between airborne microbes and health.

It had long been hypothesized that indoor microbial exposures were related to the exacerbation and even development of illness. Perhaps the earliest account was from the English physician Sir John Floyer who in 1726 reported on the development of severe asthma in a patient who had just visited a wine cellar. Although Floyer is much better known today for introducing to medicine the measurement of the pulse.

But the scientific investigation of microorganisms in indoor air really began with this 1887 paper, described in a US EPA publication as "ground breaking in the field of indoor air quality". The paper entitled "The Carbonic Acid, Organic Matter, and Micro-organisms in Air, more especially of Dwellings and Schools" was written by Carnelley (a chemistry Professor), Haldane and Anderson.



IV. *The Carbonic Acid, Organic Matter, and Micro-organisms in Air, more especially of Dwellings and Schools.*

By Professor Thos. Carnelley, *D.Sc., and* J. S. Haldane, *M.A., M.B., University College; and* A. M. Anderson, *M.D., Medical Officer of Health, Dundee.*

Communicated by Sir Henry E. Roscoe, *F.R.S.*

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In this investigation in Dundee, a Scottish mill town, air was drawn at 330 mL min-1 through long glass tube containing a nutrient medium. The tube was then incubated at room temperature for colonies to develop. It was established that outdoor air contained nearly 3 times more bacteria than fungi in still places, and nearly 14 times more in open streets during dry, windy weather. The microbial counts in streets were greater during day than at night, when there was little disturbance.

For sampling indoor air, Carnelley and his colleagues visited homes without warning between midnight and 4 AM (when there was least disturbance) so that no special ventilation or other preparations had been made.

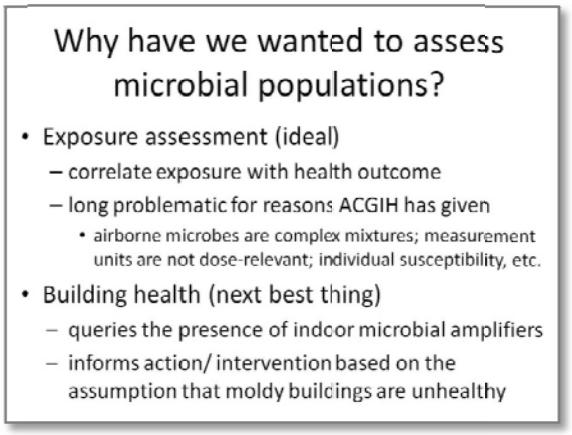
The microbial counts were found to increase with occupancy, increase in dirty houses, and decline with the amount of cubic space available per person. This last point is especially true for bacteria. These investigators also recognized that because colonies can arise from clumps of cells as well as single cells the numbers should be expressed as colony forming units (CFUs), rather than cells or spores. Their studies stand comparison against those in the many published papers which have appeared since.

Mean CFU m^{-3} air (range)	1-room houses	2-room houses	4-room houses
Bacteria	58,000 (6,000-120,000)	43,000 (6,000-118,000)	8,500 (500-16,000)
Fungi	1,200 (0-5,000)	2,200 (0-10,000)	400 (0-1,000)

In a separate investigation of schools, Carnelley and his co-workers observed greater counts for boys' classes than for girls, attributing this to boys being more restless and less hygienic than girls, and found that a class of boys boosted bacterial counts 15 times. And they attributed elevated counts during chemistry lectures to student applause.

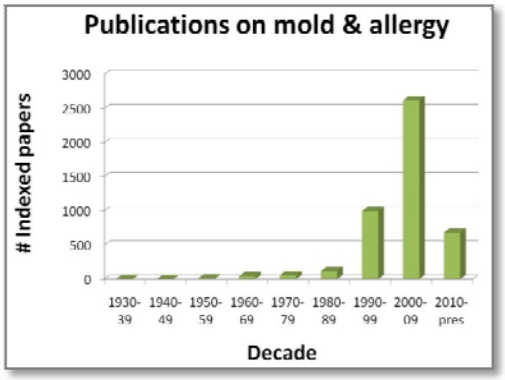
Since 1887, the many hundreds of investigations of indoor air that have been carried out using more sophisticated sampling equipment have confirmed the basic findings of these early studies by Carnelley and his colleagues. A great deal of effort has been spent on the investigation of indoor mold growth and how it may affect health. As a result, a vast amount has been learned about the identity of the fungi and bacteria in the built environment, and about their allergenic and toxic characteristics that may impact on the respiratory health of building occupants. However, assessing the actual exposure of occupants to airborne microbes continues to be a particular problem.

We desire methods that can provide accurate exposure assessment, but for many reasons, our currently methods fall short. The next best thing is to apply our imperfect methods to the assessment of building condition, and based on the assumption that moldy buildings are bad, we use the information that is obtained to inform our actions.



Part II – Where are we

In the 1920s, the Dutch physician Storm van Leeuwen hypothesized a link between environmental exposures and asthma by studying differences in allergic sensitivity among normal and asthmatic patients. From that point, relatively little advancement was made in aerobiology as it related to health until the 1950s and 1960s when the impact of environmental molds on health became an active area of research.



But it is only in the last 3 decades that the dramatic rise in prevalence of asthma and allergy in children has driven an unprecedented level of interest in this area, as is reflected by the surge in publications on mold and allergy in the scientific and medical literature over these last few decades.

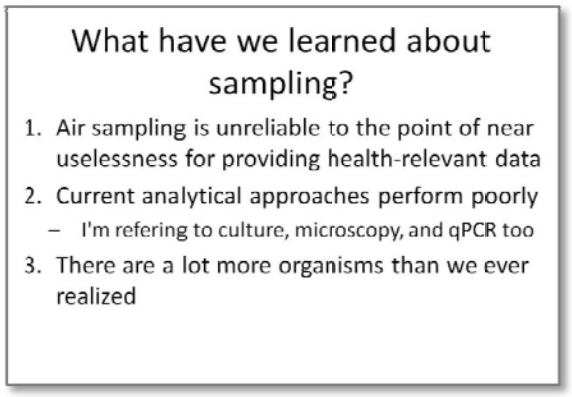
So what then have we learned about molds and health in this time?

We know that allergy and asthma is now epidemic in children in westernized countries. This is new. And genetic predisposition predicts only half of those who develop disease. In the other half of asthmatics, their disease appears to have an environmental basis. In this latter group, it is thought that exposures in early life are most influential in determining who goes on to develop disease and who doesn't. But we still don't know a great deal about what exactly those influential exposures are.

Few large population health studies have included environmental measurement, relying instead upon occupant reported observations of mold and dampness, leakage, and so on. Only several dozen very valuable large cohort studies have included objective exposure measures combined with careful inspection of buildings. From these and other smaller-scale investigations, we know that children who live in moldy houses are sicker, and that complex interactions between their genes and their environmental exposures (so here I'm referring to microbes both from the built environment and the "human microbiome" – that bunch of microbes that lives in and on us) coupled with environmental chemicals, air pollution, and so on, influence who gets sick and who doesn't.

We have also learned a great deal about sampling in the days since Pierre Miquel – and what we've learned hasn't been all that reassuring.

1) We've learned that air sampling is unreliable almost to the point of uselessness for providing health-relevant exposure information. 2) We've learned that the current analytical approaches perform poorly in terms of allowing us to reconstruct the microbiological composition of the environment that was sampled. And this includes the techniques we use most commonly, culture and microscopy, but I'd include species-specific PCR-based techniques here too. 3) And lastly, we've learned, with both excitement and disappointment, that there are far more microorganisms on our planet than we thought.



1) Exposure assessment

So in the context of population health research, given that we now know air sampling is not helpful in understanding our exposures to microbes in the built environment, how do we sample the environment to forecast health-relevant exposures in a qualitatively and quantitatively meaningful way?

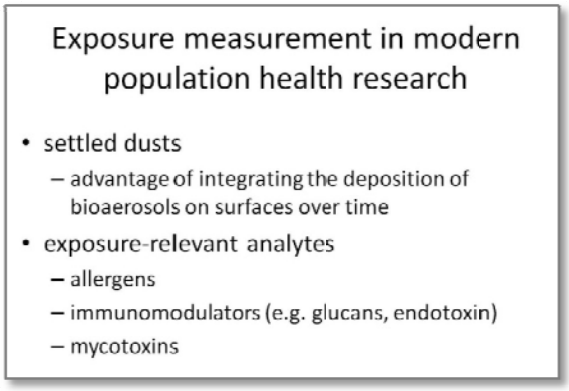
Like Darwin and Ehrenberg, we study the dust. Dust that accumulates in the built environment. And just like the deck of the Beagle, indoor dust gathers particles that settle out from the air. Dust captures the periodic bursts of particles, integrating these events into a continuous record. But dust not only serves as the sample, it also serves as the source of airborne particles – particles that become resuspended in the air in response to human activity.

In this study by Ferro and co-workers, the generation of fine particles was measured in response to various activities and used to develop a set of decay models. By subtracting the concentration of fine airborne particles generated by each prior activity from the subsequent decay curve, they were able to estimate the impact of each single activity on the release of airborne particles. Similar work has been conducted by colleagues here at UNLV led by Professor Linda Stetzenbach.

Collectively, these studies paint a picture of how we become exposed to particles in the built environment, and it looks something like this.

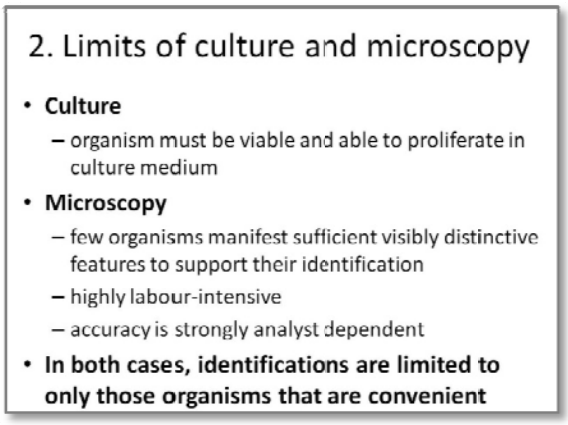
Just like Pigpen, as we go about our day-to-day lives, we are continuously surrounded by a shell of particles. These particles originate from many places, but their principle source and sink is dust. This was articulated in the 2004 review by the Institute of Medicine on Damp Indoor Spaces and Health.

So in order to monitor exposures in the built environment, most modern population health research studies collect



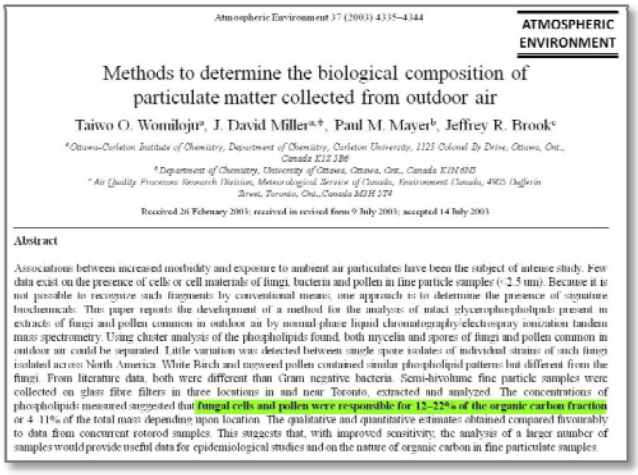
dust and measure it for the presence and concentration of a range of health-relevant contaminants, including allergens, immunomodulatory chemicals like endotoxin, beta-glucan and mycotoxins.

2) Limits of culture and microscopy



The traditional techniques of culture and microscopy may in some cases allow us to infer the presence of these health-relevant contaminants, but these methods don't allow us to measure the materials directly. Nor do these traditional methods let us accurately reconstruct the microbial communities that are present. This is because far too many organisms get missed. In the case of fungi, we generally lump together all of the culturable yeasts (which turn out to be really important health modifiers and often provide useful clues to IAQ problems); we miss most members of the basidiomycota and the more fastidious members of the ascomycota; and we can't identify anything that fails to produce spores or sufficiently diagnostic structures when it grows. And in the case of the bacteria, only the most hardy heterotrophs are detected, and then only by culture. And our identification of these organisms usually amounts to no more than a best guess.

Each of these traditional techniques, culture and microscopy is fatally biased. Culture can only detect organisms that are alive and able to grow in the nutrient media they are provided. And microscopy can only recognize those few organisms with distinctive features, and even then it is very labour-intensive and utterly dependent on the knowledge base of the analyst, not the laboratory. What happens then is that we only identify those very few organisms that are convenient. And for the purposes of evaluating the building condition, this is often enough. But as a way to understand the health implications of indoor microbial exposures, it is entirely insufficient.


Atmospheric Environment 37 (2003) 4335–4344

ATMOSPHERIC ENVIRONMENT

Methods to determine the biological composition of particulate matter collected from outdoor air

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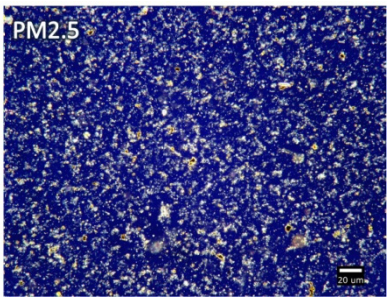
Abstract

Associations between increased morbidity and exposure to ambient air particulates have been the subject of intense study. Few data exist on the presence of cells or cell materials of fungi, bacteria and pollen in fine particle samples (< 2.5 μm). Because it is not possible to recognize such fragments by conventional means, one approach is to determine the presence of signature biochemicals. This paper reports the development of a method for the analysis of intact glycerophospholipids present in extracts of fungi and pollen common in outdoor air by normal phase liquid chromatography/electrospray ionization tandem mass spectrometry. Using cluster analysis of the phospholipids found, both mycelia and spores of fungi and pollen common in outdoor air could be separated. Little variation was detected between single spore isolates of individual strains of such fungi isolated across North America. White Birch and ragweed pollen contained similar phospholipid patterns but different from the fungi. From literature data, both were different than Gram negative bacteria. Semi-hivolume fine particle samples were collected on glass fibre filters in three locations in and near Toronto, extracted and analyzed. The concentrations of phospholipids measured suggested that fungal cells and pollen were responsible for 12–22% of the organic carbon fraction or 4–11% of the total mass depending upon location. The qualitative and quantitative estimates obtained compared favourably to data from concurrent rotorod samples. This suggests that, with improved sensitivity, the analysis of a larger number of samples would provide useful data for epidemiological studies and on the nature of organic carbon in fine particulate samples.

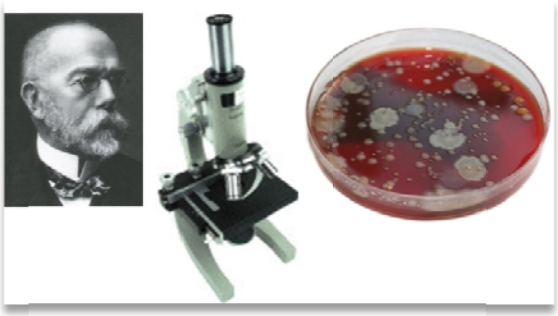
A further complexity arises when we think about that portion of biological particles in the air that are fragmented. Indeed it turns out that most of the biological particles in the air are not whole cells but fragments, and they are abundant in the air. This paper written a decade ago by Womiloju and co-workers found that nearly a quarter of the fine organic carbon mass in the outdoor air consisted of cell debris from a wide range of organisms.



And what it looks like. We inhale these particles. From work done by my former postdoc Dr. Brett Green and others, we now suspect that these fine, broken-down particles are responsible for what amounts to our most immunologically significant bioaerosol exposures. But we still can't measure these particles using traditional analytical methods.

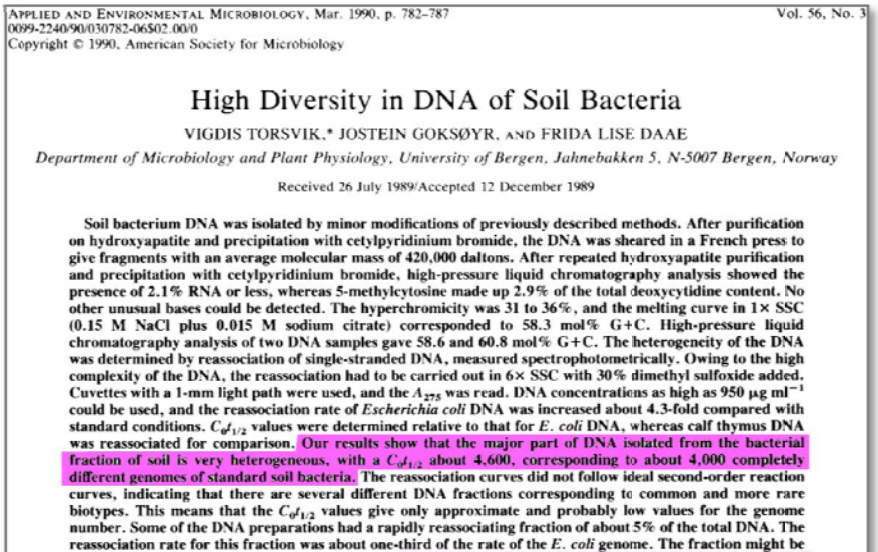
3) Lots more species than we thought

The third thing we've learned about sampling relates to the state of our knowledge about the earth's microbial biodiversity – and it turns out to be surprisingly poor.



You'll recall our earlier discussion about Louis Pasteur. Pasteur was the father of microbiology. In the mid 19th century he taught us to grow microbes in Petri dishes and look at them under the microscope, and that's mostly how we've been studying microbes ever since. Or at least until relatively recently.

As an example, one of the very best studied microbial systems over the past century has been soil. Scientists have studied soil microbiology extensively to understand nutrient cycling and to learn about the lifecycles of plant pathogens. Microbiology has used soil as a kind of model habitat. Probably every undergraduate student of microbiology at one time or another performed serial dilution studies of soil to count and identify the bacteria and fungi living in it. As a result, thousands of microbes have been reported from soil. Whole books have been written on it.



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High Diversity in DNA of Soil Bacteria

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Soil bacterium DNA was isolated by minor modifications of previously described methods. After purification on hydroxyapatite and precipitation with cetylpyridinium bromide, the DNA was sheared in a French press to give fragments with an average molecular mass of 420,000 daltons. After repeated hydroxyapatite purification and precipitation with cetylpyridinium bromide, high-pressure liquid chromatography analysis showed the presence of 2.1% RNA or less, whereas 5-methylcytosine made up 2.9% of the total deoxycytidine content. No other unusual bases could be detected. The hyperchromicity was 31 to 36%, and the melting curve in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) corresponded to 58.3 mol% G+C. High-pressure liquid chromatography analysis of two DNA samples gave 58.6 and 60.8 mol% G+C. The heterogeneity of the DNA was determined by reassociation of single-stranded DNA, measured spectrophotometrically. Owing to the high complexity of the DNA, the reassociation had to be carried out in 6× SSC with 30% dimethyl sulfoxide added. Cuvettes with a 1-mm light path were used, and the A_{275} was read. DNA concentrations as high as 950 μg ml^{-1} could be used, and the reassociation rate of *Escherichia coli* DNA was increased about 4.3-fold compared with standard conditions. $C_0t_{1/2}$ values were determined relative to that for *E. coli* DNA, whereas calf thymus DNA was reassociated for comparison. Our results show that the major part of DNA isolated from the bacterial fraction of soil is very heterogeneous, with a $C_0t_{1/2}$ about 4,600, corresponding to about 4,000 completely different genomes of standard soil bacteria. The reassociation curves did not follow ideal second-order reaction curves, indicating that there are several different DNA fractions corresponding to common and more rare biotypes. This means that the $C_0t_{1/2}$ values give only approximate and probably low values for the genome number. Some of the DNA preparations had a rapidly reassociating fraction of about 5% of the total DNA. The reassociation rate for this fraction was about one-third of the rate of the *E. coli* genome. The fraction might be a population of plasmids and/or bacteriophages. Our results indicate that the diversity of the total bacterial community in a deciduous-forest soil is so high that diversity indices based on DNA heterogeneity can be determined only with difficulty. Most of the diversity is located in that part of the community which cannot be isolated and cultured by standard techniques.

Just as I was finishing my undergraduate degree in the late 1980s, a group in Norway published this paper investigating the bacterial diversity of soil not using microscopy or culture, but rather, using DNA. They isolated whole genomic DNA from the community of organisms in a single gram of soil, they melted all of the DNA strands apart using heat, and then they followed the temperature profile as the separated strands of DNA reassociated with each other. Their findings were groundbreaking at the time. The experiment suggested that the soil sample contained upwards of 4,000 different species, and that the vast majority of the diversity in the sample was contributed by microbes that could not be cultured.

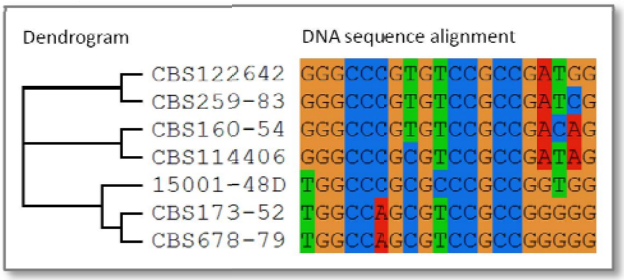
Other workers further refined this technique hinting at the possibility of millions of different microbes inhabiting a mere thimble full of soil. These experiments frightened many microbial ecologists who believed at the time that the knowledge of these well-studied systems was largely complete.

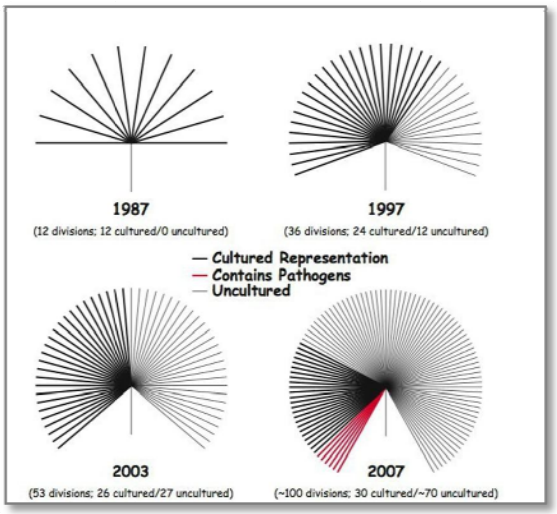
These methods succeeded in demonstrating an important gap in knowledge; however they also had significant limitations because they were unable to identify precisely what those multitude of organisms actually were.

Around the same time, a group from University of Colorado at Boulder led by Norman Pace developed the idea of amplifying a single, diagnostic gene region from all organisms in a whole community sample using the newly developed technique of polymerase chain reaction – PCR – as a way to study the microbial diversity of a hot spring.

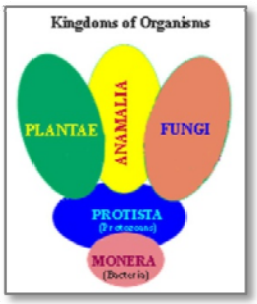
Pace's group then cloned the amplified fragments into bacterial cells, one fragment per cell, and prepared essentially a bacterial library that contained the complete diversity of that gene region from the whole population. Pace then sequenced the diagnostic gene from each of his bacterial clones, and lined up the sequences, one by one, in a way that allowed him to determine the genetic similarity of the various organisms they originated from.

Pace's tree looked something like this, except a great deal larger, where those organisms that have greater similarity in the particular gene region tested are situated more closely to each other on this branching diagram. Then, by inserting the gene sequences of known organisms into the matrix, he was able to infer the identification of the organisms that were originally present in the hot spring water. Many, it turned out, did not match up with anything that was known to science.





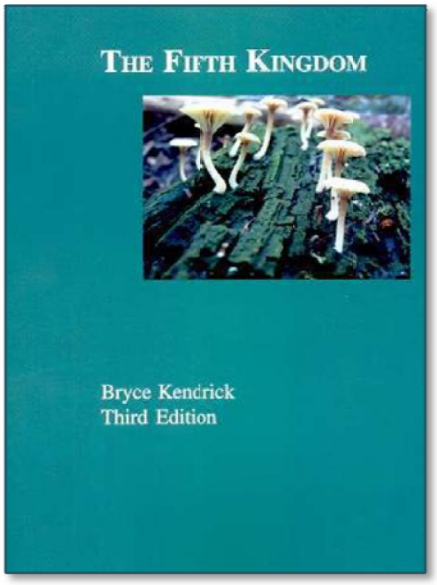
When Pace began his work in 1987, there were 12 divisions in the bacteria, all of which were known from culture. As Pace and others continued to employ these newly-honed culture-independent methods, what emerged was a picture of bacterial biodiversity that included a great many more divisions of bacteria than were formerly known. Today we recognize over 100 divisions two thirds of which cannot be cultured.



This phenomenon is not limited to bacteria but is true of microbes in general. When I was an undergraduate student, I was taught that living things could be classified into 5 kingdoms: Monera, Protista, Plants, Animals and Fungi, following the higher classification of life outlined by Robert Whittaker in the late 1960s.

And still today, the most popular textbook on mycology written by my friend and colleague Bryce Kendrick, by its title, *The Fifth Kingdom*, tips its hat to Whittaker. But now, with the advent of molecular genetic methods to poll nature for undiscovered diversity and to determine relationships between organisms in a

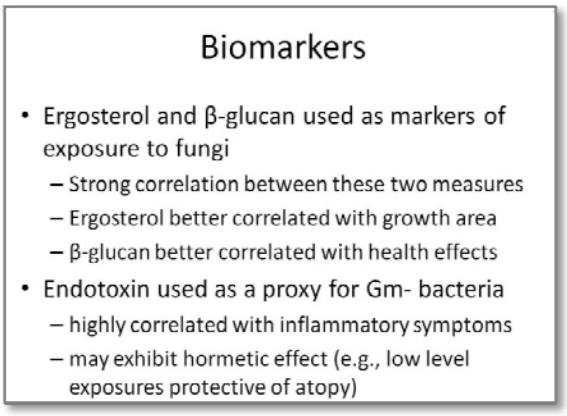
manner that is unbiased by their form and function, estimates of the number of these highest level domains of life range from tens to hundreds. A recent paper by Roger and Simpson in Current Biology (19: R165–R167, 2009) has even suggested that this rapid rate of scientific advancement makes it nearly impossible to produce a meaningful estimate of higher-level taxonomic groupings.



All this may not be much comfort to you, but I can assure you that these are exciting times to be a biologist.

Biomarkers

In terms of indoor mould assessments, culture and microscopy have seen their day. But looking beyond these techniques, what options remain? Well I alluded earlier to the fact that exposure measurement in most modern population health research relies on biochemical markers. And several non-specific biomarkers are routinely used as microbial surrogates, especially in the analysis of dust.



For fungi, both ergosterol and beta-glucan have proven to be useful exposure indicators. Ergosterol is the principal membrane sterol in fungi – the equivalent of cholesterol in animals. All fungi have ergosterol, and in roughly comparable quantities. The concentration of ergosterol in a sample strongly predicts the amount of fungal biomass present. The ergosterol content of indoor dust is very well correlated mold-affected area in a building.

Beta-glucan is a cell-wall polysaccharide present in many fungi (with exceptions) and in some plants and animals. Although it is not as specific to fungi as ergosterol, glucan itself is a health relevant contaminant because it elicits the inflammatory response through innate immune mechanisms. In other words, you develop inflammation when exposed to glucan whether or not you have allergies. Glucan and ergosterol correlate very well with each other, however of the two, glucan content is better correlated with health effects, as you might expect.

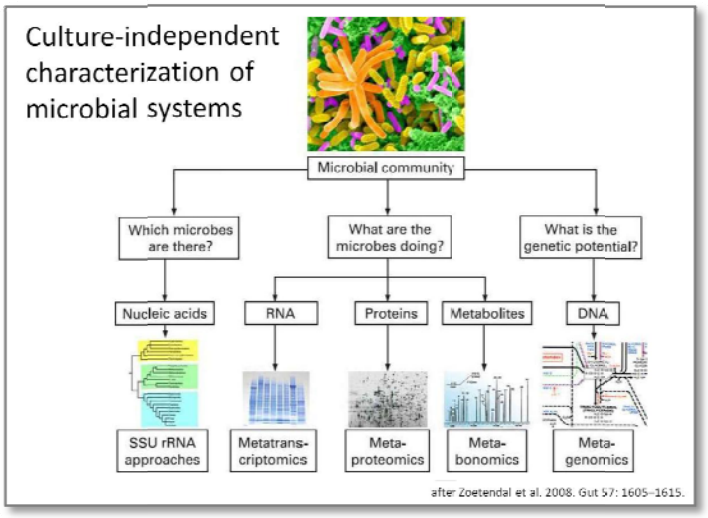
Endotoxin is a lipopolysaccharide present in most gram negative bacteria. It is uniquely bacterial, and thus a good marker of the presence of gram-negative bacteria. Like beta-glucan, endotoxin is a powerful inflammatory mediator and has been shown to potentiate allergic sensitization and asthma. Paradoxically though, low level exposures to endotoxin in young children have been found to exert a protective effect against allergic sensitization. The mechanism of this effect remains unknown.

Altogether, however useful these biomarkers have been in resolving relationships between indoor exposures and health, none of them offer information on the identity of the organisms responsible for their production, perpetuating a key gap in our understanding the microbial ecology of the built environment.

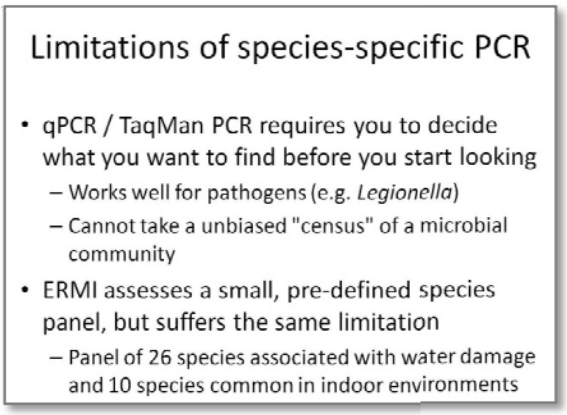
Other more specific biomarkers such as allergens are being used in these types of studies, and have been for many years. And as discussed by Dr. Eva King in yesterday's session, modern methodological approaches are tremendously improving the value of these measures as well.

Culture-independent methods

At the beginning of this talk, I suggested that during the past decade, we have been experiencing a sea change in how we do microbiology. That shift has been brought about by our long-overdue recognition of the limits of the Pasteurian tradition of culture and microscopy. And it has been facilitated by rapid technological advancements in our ability to characterize both genes and gene products such as proteins and metabolites.



So far, the only modern culture-independent methods to be widely adopted by the aerobiology community have been PCR-based tests for specific microorganisms. Variations of species-specific PCR have held the promise of being very powerful, highly sensitive and less influenced by interanalyst variability than traditional approaches.

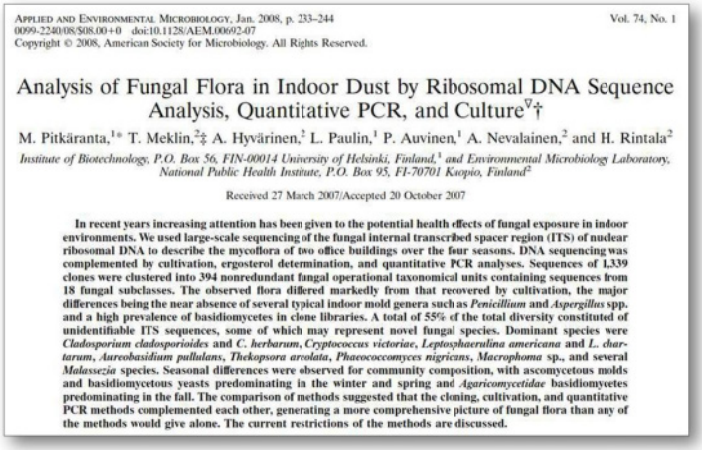


However, early adopters of these technologies will testify to their analytical limitations, and the challenges around developing awareness and acceptance of these methods among the various user communities.

Species-specific PCR tests require you to decide what you want to find before you start looking for it. This paradigm works well for certain microbes, particularly pathogens like *Legionella*. However, it does not permit an unbiased census of a microbial community to be taken. Test panels that include multiple species-specific assays, like ERMI, have improved on this limitation somewhat, but they are still restricted by their ability to detect only those species whose identity has been pre-determined. Unanticipated or unknown microbes are skipped as a consequence.

And it is becoming increasingly clear that we do not yet know enough about building microbiology to determine what organisms can be skipped without losing essential information.

In her doctoral thesis, Miia Pitkaranta, under the supervision of Aino Nevalainen and Helena Rintala at the University of Helsinki applied an approach to the characterization of fungi in indoor dust similar to that used by Norman Pace and his colleagues to study hot spring bacteria.


APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2008, p. 233–244 Vol. 74, No. 1
0099-2240/08/$08.00+0 doi:10.1128/AEM.00692-07

Analysis of Fungal Flora in Indoor Dust by Ribosomal DNA Sequence Analysis, Quantitative PCR, and Culture▽†

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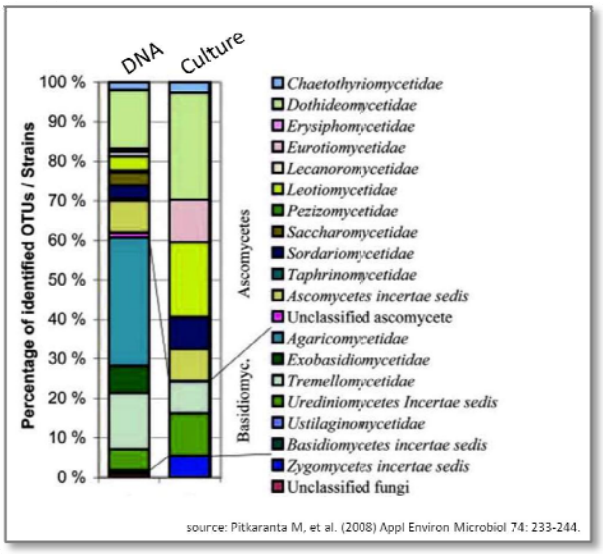
In recent years increasing attention has been given to the potential health effects of fungal exposure in indoor environments. We used large-scale sequencing of the fungal internal transcribed spacer region (ITS) of nuclear ribosomal DNA to describe the mycoflora of two office buildings over the four seasons. DNA sequencing was complemented by cultivation, ergosterol determination, and quantitative PCR analyses. Sequences of 1,339 clones were clustered into 394 nonredundant fungal operational taxonomical units containing sequences from 18 fungal subclasses. The observed flora differed markedly from that recovered by cultivation, the major differences being the near absence of several typical indoor mold genera such as *Penicillium* and *Aspergillus* spp. and a high prevalence of basidiomycetes in clone libraries. A total of 55% of the total diversity constituted of unidentifiable ITS sequences, some of which may represent novel fungal species. Dominant species were *Cladosporium cladosporioides* and *C. herbarum*, *Cryptococcus victoriae*, *Leptosphaerulina americana* and *L. chartarum*, *Aureobasidium pullulans*, *Thekopsora areolata*, *Phaeococcomyces nigricans*, *Macrophoma* sp., and several *Malassezia* species. Seasonal differences were observed for community composition, with ascomycetous molds and basidiomycetous yeasts predominating in the winter and spring and *Agaricomycetidae* basidiomycetes predominating in the fall. The comparison of methods suggested that the cloning, cultivation, and quantitative PCR methods complemented each other, generating a more comprehensive picture of fungal flora than any of the methods would give alone. The current restrictions of the methods are discussed.

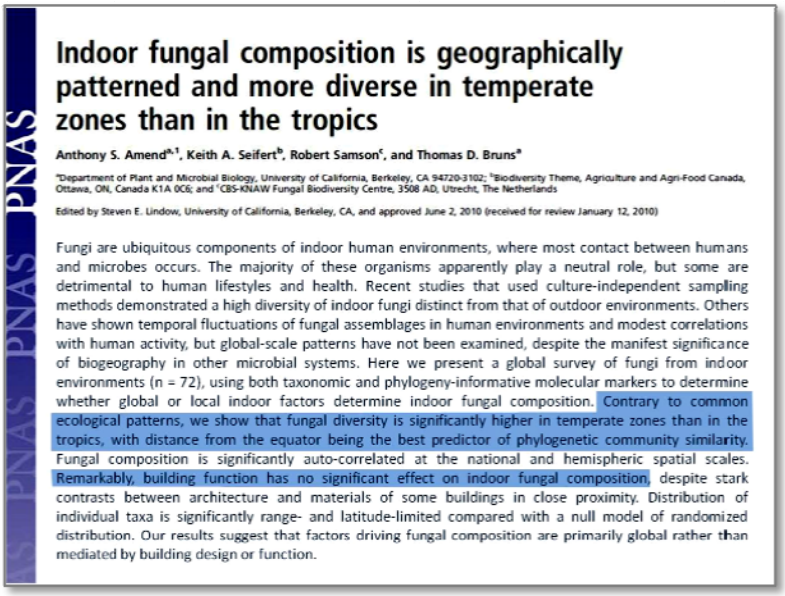
In their initial study, they collected indoor dust from two buildings in Finland at four time points over a 1 year period. They divided each aliquot of dust into two parts, culturing one and subjecting the other, like Pace, to a very painstaking and labour-intensive culture-independent cloning and sequencing analysis.



And what they found was just as shocking as you might expect. Overall, their cultures from the two buildings yielded 37 species – not many more than most of you might be accustomed to seeing on a laboratory report.

The DNA-based method, by contrast, yielded nearly 400 different species. At least half of these species represented subclasses of fungi that were completely absent in the culture-based studies.

Sixty percent of the taxa found by the DNA-based method were members of the basidiomycota, compared to fewer than 20% in the culture assay. And nearly a fifth of the dust-borne species detected by DNA were human-associated lipophilic basidiomycete yeasts, responsible for a range of skin conditions from liver spots to dandruff. This is the very same group of fungi shown by Dr. Storm van Leeuwin in the 1924 paper I mentioned earlier to be uniformly potent allergens in all the asthmatics he tested. But until Pitkaranta's work, no one had any idea that they were among the most common fungi in indoor dust. As well, they are one of the few fungal groups that produce beta-1,6 rather than 1,3 linked glucans.



Indoor fungal composition is geographically patterned and more diverse in temperate zones than in the tropics

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Edited by Steven E. Lindow, University of California, Berkeley, CA, and approved June 2, 2010 (received for review January 12, 2010)

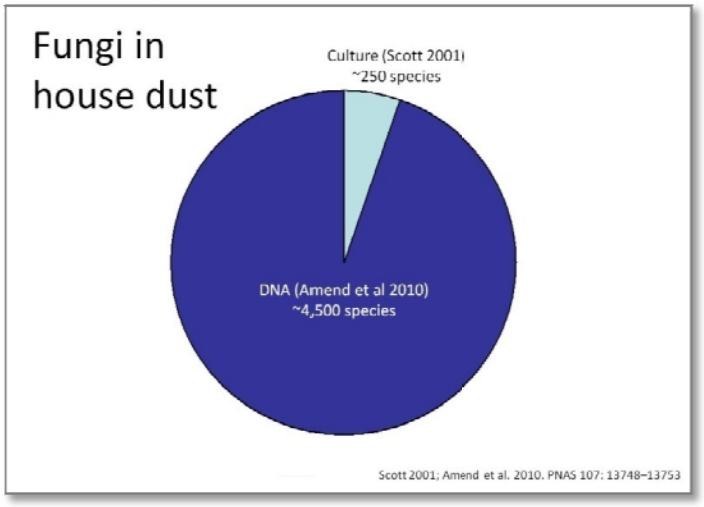
Fungi are ubiquitous components of indoor human environments, where most contact between humans and microbes occurs. The majority of these organisms apparently play a neutral role, but some are detrimental to human lifestyles and health. Recent studies that used culture-independent sampling methods demonstrated a high diversity of indoor fungi distinct from that of outdoor environments. Others have shown temporal fluctuations of fungal assemblages in human environments and modest correlations with human activity, but global-scale patterns have not been examined, despite the manifest significance of biogeography in other microbial systems. Here we present a global survey of fungi from indoor environments (n = 72), using both taxonomic and phylogeny-informative molecular markers to determine whether global or local indoor factors determine indoor fungal composition. Contrary to common ecological patterns, we show that fungal diversity is significantly higher in temperate zones than in the tropics, with distance from the equator being the best predictor of phylogenetic community similarity. Fungal composition is significantly auto-correlated at the national and hemispheric spatial scales. Remarkably, building function has no significant effect on indoor fungal composition, despite stark contrasts between architecture and materials of some buildings in close proximity. Distribution of individual taxa is significantly range- and latitude-limited compared with a null model of randomized distribution. Our results suggest that factors driving fungal composition are primarily global rather than mediated by building design or function.

Even more recently, Amend and co-workers applied a powerful high-throughput DNA pyrosequencing method to study fungi in 72 diverse indoor environments world-wide in an effort to establish commonalities in the indoor fungal microbiome. Their results were equally shocking. In contrast to the common ecological pattern where life is most diverse at the equator, these workers found that precisely the opposite is true of indoor fungi, their biodiversity actually increases at greater distances from the equator.

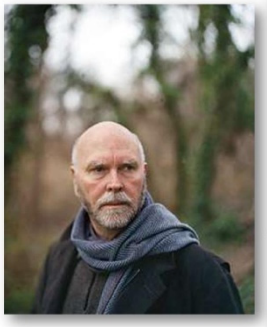
It might be expected that this is a function of increased constraints on energy utilization, and accordingly the construction of ever tighter, more controlled buildings. Another interesting finding from this work was the lack of dependency of fungal diversity in dust on the building function, architectural design or materials composition. Still, with a sample size of 72, it is unlikely that these findings are definitive, but they are certainly tantalizing.

I completed my doctoral work over a decade ago studying fungi in dust from 400 homes in Canada using culture techniques. It was part of a large population health study on the early-life environmental

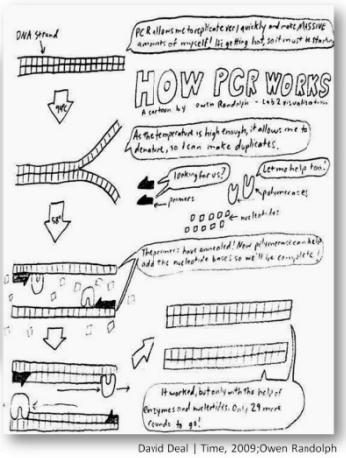
determinants of respiratory disease, and the work was quite detailed and painstaking. During my defence, one of my examiners commented that my work was surely to provide the last word on the fungal biodiversity of dust. She couldn't have been more wrong. My studies revealed what seemed then to be an impressive list of over 250 species. But in their study of only 72 buildings, Amend and co-workers found 18 times that number. And even then, their measured diversity was probably underestimated by 20% or more.



Part III – Where we're going



J. Craig Venter



In 2003, Craig Venter, the developer of PCR and the Einstein of our story, issued a $500,000 industry challenge to develop the capability to sequence an entire human genome for under $1000. Up until last year, many industry watchers calmly denounced that this goal was even remotely achievable.

In 2011 Venter's challenge was merged with the $10 million Archon X-Prize for the first group able to design a platform capable the lofty goal of sequencing 100 human genomes in 30 days or less.

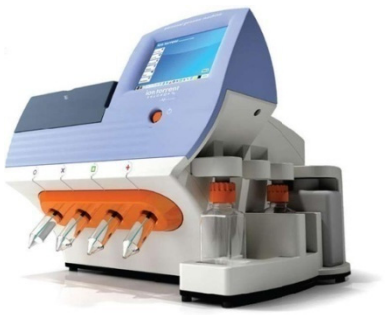
What once seemed impossible is now on the brink of probability, and in a very short time frame.

Next-generation high throughput DNA sequencing

In 2005 Stephan Schuster at Penn State University and his colleagues published the first sequences of an environmental sample generated with high-throughput DNA sequencing, in this case massively parallel pyrosequencing developed by 454 Life Sciences, now a subsidiary of Roche Diagnostics. This pyrosequencing method was the technique used by Amend and co-workers in their study of dust-borne fungi.

The use of high throughput DNA sequencing to characterize microbial communities has come to be known as metagenomics, and it places high demands both on the sequencing technology as well as computational data processing.

Initially the costs of high throughput sequencing methods were high, in the 10s of thousands of dollars per reaction. However with recent technological advancements, we have seen an exponential reduction in costs of these methods while at the same time their power has increased enormously.

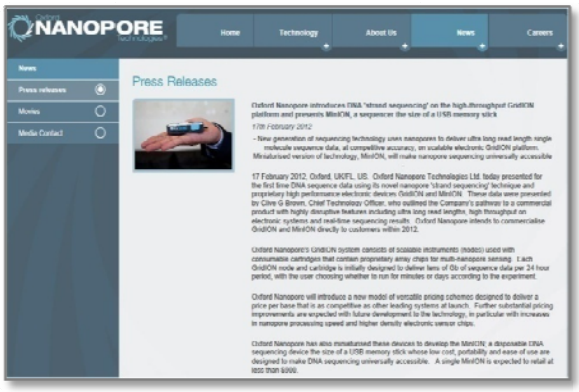


As one example of this rapid pace of technology, the Ion Torrent platform was launched in 2010, and the company was recently acquired by global biotech giant Life Technologies for three quarters of a billion dollars.

Currently with this platform, it is possible to characterize the entire fungal or bacterial community exhaustively of up to 80 samples simultaneously at a cost of around $200 per sample. It is likely that in the next 2 years, the cost may be reduced to as low as $20 per sample. Remember, this is not just counting spores or CFUs, this counts and identifies everything, regardless of what it is, all identified to the prevailing limits of science.

Access to this sort of sequencing power used to be limited to large, wealthy academic laboratories. This device is worth about $75,000. This new family of sequencing platforms have democratized DNA sequencing technology to the point that it is now accessible to nearly every laboratory.

This means no more using E. coli as a proxy for fecal pathogens. Instead you simply look for all the pathogens themselves. Don't know what pathogens to look for? Doesn't matter. It also means no more arguments between analysts about whether a spore looks like *Alternaria* or *Ulocladium*. And all yeasts and wood rot fungi and otherwise unknown and underrepresented things along with everything else can be characterized – all in a single test.



And it gets even better. Two weeks ago, at the Advances in Genome Biology and Technology Conference on San Marco Island, Florida, the UK-based Oxford Nanopore Technologies unveiled a single strand DNA sequencing platform the size of a USB memory stick that is capable of delivering tens of gigabases of DNA sequence in a 24 hour period at a cost of less than $900.

And just like a USB memory stick, you actually plug the device into the USB port of a laptop computer and it runs directly on the laptop in real time. In theory, if this platform meets expectations, it would bring us close to that $20 per sample threshold I was talking about. And that's basically now.

In the meantime, the two leading developers, Roche and Illumina, have both announced that their technologies will likely meet the X-Prize goals by the end of 2012.

So although I'm supposed to be describing the future here, I guess my message is that the future is now.

How will these technologies be brought to bear on the study of indoor microbes?

Four years ago the Alfred P. Sloan Foundation developed a major research funding initiative targeting the microbiology of the built environment. The goal of the program is to bring the use of these cutting edge technologies to the investigation of the indoor environment, and to establish a knowledge base to understand the implications of the data they produce, both in terms of health as well as building condition.

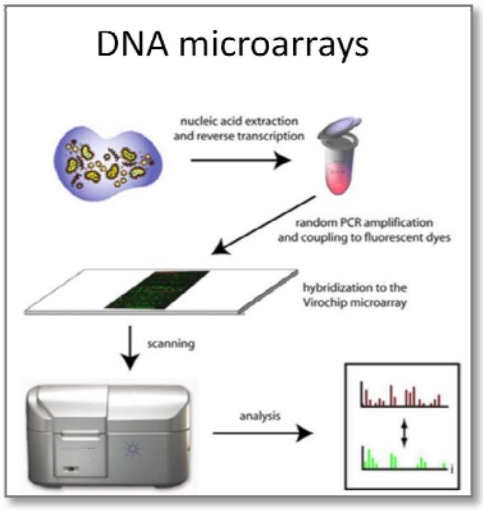
To date, Sloan has released nearly $10 million in research grants. I spoke earlier of the Amend et al study

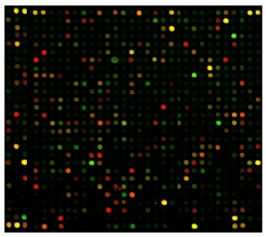
which was one of the initial pilot studies funded through this program. A number of other large-scale projects are currently underway that will provide a much more detailed look at the indoor microbiome than ever before.

Over the next 10 years, I fully expect that these and other methods will completely displace the albatross of the Petri dish and microscope, that together have done as much to advance the study of microbiology as they have to impede it.

DNA microarrays

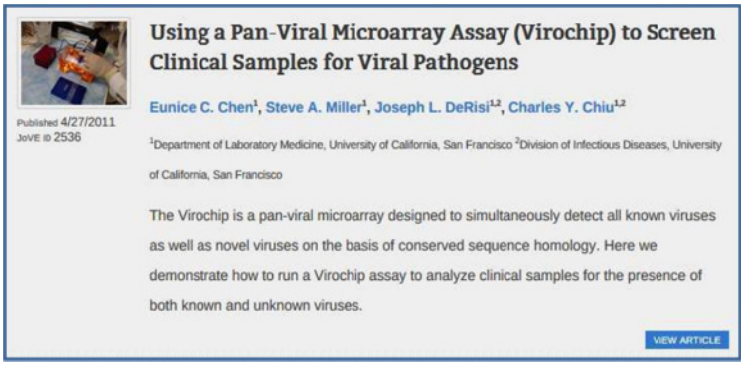
DNA microarrays are another technology that continues to offer promise as an inexpensive and rapid diagnostic tool. Microarrays are essentially microscope slides onto which a DNA matrix has been printed, using a device much like an inkjet printer. Each spot in this matrix represents a distinct organism. For increased specificity, each organism is normally represented by multiple spots. A given matrix can be printed with thousands of spots.





When the microarray is treated with an extract from a sample, where there is a match, the DNA from the sample binds to a spot. By reading the intensity with which DNA from the sample has bound to the matrix, it is possible to determine the microbiological community composition of the sample.

This method has been used successfully in the clinical detection of viruses, and is the basis of a microarray platform called the ViroChip. It has been adapted to work with double stranded and single stranded DNA and RNA viruses. And the current version of the ViroChip is capable of accurately detecting and identifying all known human viruses.



Think about that for a second: all known viruses. In a single test.

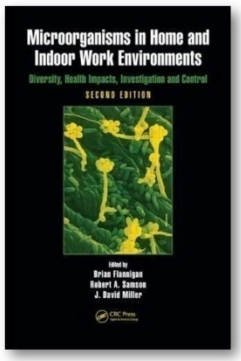
And this test is in use already in clinical laboratories, and it costs less than $100 per sample.

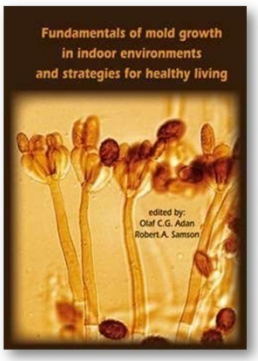
In conclusion, we have discussed that the tradition of aerobiology is long and rich, but our reliance on outdated tools has stagnated discovery. The traditional analytical tools of aerobiology are obsolete. I predict that culture and microscopy will largely be replaced over the next 10 years by methods that provide a much more objective and comprehensive picture of the microbiology of the built environment. New approaches will yield a much clearer understanding of the indoor microbiome as a determinant of health. Armed with a greater understanding of the important exposures, it will be possible to propose much more strategic and effective interventions.

Those of us with an interest in indoor microbiology must adapt our methods or, like Darwin's Galapagos finches, we risk extinction.

Acknowledgements

I thank Professors Brian Flannigan, J David Miller and Richard Summerbell for numerous conversations leading to many of the ideas presented in this talk; however, I take full responsibility for any errors. Professor Flannigan prepared the text dealing with the facinating studies of Carnelley et al., and we co-presented this material in addition to several other elements of this talk recently in Amsterdam at a symposium sponsored by the Royal Netherlands Academy of Arts and Sciences. This topic has also been the subject of chapters published recently in two books, *Microorganisms in Home and Indoor Work Environments*, 2nd edition (Flannigan, Samson & Miller, CRC Press, 2011); and *Fundamentals of Mould Growth in Indoor Environments* (Adan & Samson, Wageningen Academic Publishers, 2011).





In finance, it is customary to provide a disclaimer on forward-looking statements such as those I have made in this presentation, to the extent that, while my predictions are speculative, they are accurate to the best of my knowledge. As well, I have no financial interest in any of the emerging technologies I have discussed today; I neither provide these services nor do I promote their use. I do, however, believe strongly that we as a community need to be aware of these very recent seismic shifts in technology, and consider how best to prepare ourselves for their certain impact on our practice in IAQ.

I thank you for your attention.

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Ross Holland, National Oceanography Centre; NASA Image courtesy Jeff Schmaltz, MODIS Land Rapid Response Team; de.wikipedia.org 2012–02–27; Ehrenberg CG. 1818. *Sylvae Mycologicae Berolinenses*; Bernard Siedlecki, Pinchin Environmental; Ehrenberg CG. 1847. Abh. Kgl. Akad. Wiss. Berlin, Bd. 269–460; Eric Condliffe, University of Leeds; Miquel MP. 1883. *Les organismes vivants de l'atmosphère*. Paris: Gauthier-Villars; Carnelley et al. 1887. The Carbonic Acid, Organic Matter, and Micro-organisms in Air, more especially of Dwellings and Schools. Philosophical Transactions of the Royal Society of London. B 178: 61–111; Publications by decade in mould and allergy/ asthma ISI Web of Science, accessed 2012-03-03 search term: (mold OR mould OR fung*) AND (allerg* OR asthma); Ferro AR et al. 2004. Environ Sci Technol 38: 1759–1764; Charles Schultz *Pigpen* (inspired by Professor J David Miller); Womilojou et al 2003. Atmospheric Environment 37: 4335–4344; Torsvik et al. 1990. Appl Env Microbiol 56: 782–787; Norm Pace Lab, UC Boulder; Kirk Harris; W. Bryce Kendrick *The Fifth Kingdom*; Zoetendal et al. 2008. Gut 57: 1605–1615; Pitkäranta et al. 2008. Appl Env Microbiol 74: 233–244; Amend et al. 2010. PNAS 107: 13748–13753; JA Scott. 2001. *Studies on Indoor Fungi*; David Deal | Time, 2009;Owen Randolph; Ion Torrent | Life Technologies; Oxford Nanopore Technologies; Chen EC et al. 2011. J Vis Exp 50: e2536, DOI: 10.3791/2536. Remaining uncredited images from Wikipedia.